Species-specific accumulation of interspersed sequences in genus *Saccharum*

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The genus *Saccharum* consists of two wild and four cultivated species. Novel interspersed sequences were isolated from cultivated sugar cane *S. officinarum*. These sequences were accumulated in all four cultivated species and their wild ancestral species *S. robustum*, but were not detected in the other wild species *S. spontaneum* and the relative *Erianthus arundinaceus*. The species-specific accumulation of interspersed sequences would correlate to the domestication of sugar canes.

Key words: domestication, interspersed sequences, *Saccharum*, Sugar cane

Saccharum is the genus of cultivated sugar canes, and belongs to the tribe Andropogoneae together with the genus *Zea* in the family Poaceae. This genus constitutes the “*Saccharum* complex” along with the genera *Erianthus*, *Miscanthus*, *Sclerostachya* and *Narenga*, all of which are considered related to the domestication of sugar cane through the generation of fertile interbred progeny (Guimarães and Sobral 1998). The genus *Saccharum* consists of six species; two wild species, *S. spontaneum* and *S. robustum*, and four cultivated species, *S. officinarum*, *S. barberi*, *S. sinense* and *S. edule* (Daniels and Roach 1987). Each species is characterized by floral characters, sugar contents, chromosome numbers and epidermal hair groups, and chromosome numbers of *Saccharum* species are characterized by both a high level of polyploidy and aneuploidy (Irvine 1999). Phylogenetic relationships in the “complex” have been investigated in molecular studies of the cytoplasmic and nuclear DNA (Al-Janabi et al., 1994; Sobral et al., 1994; Guimarães et al., 1997; Ming et al., 1998; Alix et al., 1999; Hodgkinson et al., 2002; Lima et al., 2002), and cytological studies on chromosomes (D’Hont et al., 1995; D’Hont et al., 1996; Alix et al., 1998; D’Hont et al., 1998; Piperidis et al., 2000; D’Hont et al., 2002; Cuadrado et al., 2004). In molecular cytological analyses, genus-specific repetitive DNA sequences have been investigated to reveal phylogenetic relationships among genera in the “complex” (D’Hont et al., 1995; Alix et al., 1998; Alix et al., 1999), and species-specific sequences are required to reveal domestication in the genus *Saccharum*. The present study describes the isolation of novel interspersed sequences and their species-specific accumulation in *Saccharum*, and discusses

the domestication of cultivated sugar cane.

Twelve accessions from eight species of sugar canes and relative species listed in Table 1 were used for this study. Genomic DNA was isolated from young leaves with a DNeasy Plant Mini System (QIAGEN, Germany) according to the manufacturer’s directions. Primer sets, RCH2CCS1 (F: TGGCC CGATC TTTCG ATGAG, R: ACTTT CATCT TTATC TAGCA), and SCM (F: ATCCG ACTAC GACGA GCGAA, R550: TGATG AGGAC ACCAC GCGTA, and R180: CAGAA CTCCA CTTTC GAGTG) were used for polymerase chain reaction (PCR) amplification by Ex Taq DNA polymerase (Takara, Japan) with 25 cycles of 94°C for 1 min, 60°C for 40 sec and 72°C for 2 min, after an initial denaturation at 94°C for 10 min. The primer set RCH2CCS1 was designed to amplify centromeric repeat-sequences based on homologous sequences between rice *RCH2* (Dong et al., 1998) and wheat *CCS1* (Aragon-Alcaide et al., 1996). Two primer sets, SCM-F and R550, and SCM-F and R180, were designed to amplify 550-bp and 180-bp sequences, respectively, which include homologous sequences between the genome of *Sugar Cane* and *Maize* (see below). Amplified DNA sequences were cloned into the pGEM-T easy vector system with the *E. coli* DH5 alpha strain (Promega, WI), isolated by miniprep with a QIAprep spin Miniprep Kit (QIAGEN), and sequenced with a CEQ2000XL (Beckman Coulter, CA) according to the manufacturers’ directions. Labeling by PCR and Southern hybridization were carried out as previously described (Nakayama et al., 2001). Chromosomal preparations for FISH were obtained by enzyme maceration from root tips after two hours treatment with a 0.05% colchicine solution at 25°C. FISH procedures were carried out as reported (Okada et al., 2000; Nakayama et al., 2004). The biotin-labeled probe was detected with Alexa 488-streptavidin (Molecular

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Table 1. Sugar canes and related species employed in this study. *

No.**	Species	Accessions
1.	<i>Saccharum edule</i>	IN95-0009
2.	<i>S. robustum</i>	IN84-111
3.	<i>S. officinarum</i>	Badila
4.		Ogasawara
5.	<i>S. sinense</i>	Ohshima
6.		Teckcha
7.	<i>S. barberi</i>	Chunnee
8.	<i>S. spontaneum</i>	Glagah Kloet
9.		SES205
10.		Ishigaki-39
11.	<i>Erianthus arundinaceus</i>	28NG7
12.	<i>Zea mays</i>	CI64

* Sugar cane plants were grown in a domed green house of NIAS at Tsukuba, Japan.

** Lane number in Fig. 2

Probes, OR) after tyramide signal amplification (Perkin Elmer, MA), and the DIG-labeled probe was detected with anti-digoxigenin-rhodamine (Roche Diagnostics, Germany) and Alexa 546 donkey anti-sheep IgG (Molecular Probes).

Three bands of PCR products, 1.5-, 2.2-, and 2.8-kbp, were obtained from genomic DNA of *S. officinarum* cv. Badila with the RCH2CCS1 primer set (data not shown). Predicted 240-bp products were amplified from genomic DNA of *O. sativa* cv. Nipponbare with this primer set. Instead of the 240-bp products, PCR amplified these products from *S. officinarum*. Sequence analysis revealed that a cloned 1.5-kbp PCR product consisted of two characteristic parts (Fig. 1A). One part is about 900-bp long and appears related to the centromeric regions, because it showed homology to the centromeric retrotransposon, CR, and the centromeric repeat-sequences in Poaceae (Jiang et al., 1996; Ananiev et al., 1998; Dong et al., 1998; Nonomura and Kurata 1999; Zhong et al., 2002; Jiang et al., 2003). The other part is about 550-bp in length and includes sequence homologous to the genome of maize, and 180-bp of this part appeared very frequently in the maize genome.

In the *S. officinarum* genome, the present status of the 550-bp sequence was investigated. PCR amplification of 550-bp sequences with the primer set SCM-F and R550, which were designed to amplify the whole of the 550-bp sequence, gave 550-bp products from genomic DNA of *S. officinarum* cv. Badila (Fig. 2A lane 3). PCR amplification of the 180-bp sequence with the primer set SCM-F and R180 yielded 180-bp, 1.0-kbp and faint 2.0-kbp products from *S. officinarum* (Fig. 2B lane 3). Sequence analysis revealed that the cloned 1.0-kbp product contained both 180-bp and 550-bp sequences, and the second

Fig. 1. Scheme of sequences of cloned PCR products. (A) 1.5-kb products obtained with the RCH2CCS1 primer set. (B) 1.0-kb products obtained with the SCM-F and R180 primer set. Nucleotide sequence data are available in the DDBJ / EMBL / GenBank databases under accession numbers AB197933 and AB197934.

Fig. 2. Species-specific accumulation of SoIS550 among the genus *Saccharum*. (A) PCR amplification with SCM-F and R550. (B) PCR amplification with SCM-F and R180. (C) PCR amplification with the 17SrDNA primer set (Nakayama et al., 2001). (D) Southern hybridization of SoIS550 in *Alu* I-digested genomic DNA. Lane numbers are described in Table 1.

180-bp sequence was included in the 550-bp sequence (Fig. 1B). Southern hybridization of the DIG-labeled 550-bp sequence in *Alu* I-digested genomic DNA of *S. officinarum* showed three strong signals at 600-bp, 1.2-kbp and 1.7-kbp (Fig. 2D lane 3). The intensity of the hybridization bands suggested that the 550-bp sequences should be repeated in the *S. officinarum* genome. FISH studies with the 550-bp sequence and centromeric SCEN repeat-sequences, amplified by PCR from *S. officinarum* with the primer set SCEN-A and SCEN-B (Nagaki et al., 1998), revealed that signals of the 550-bp sequence were interspersed among *S. officinarum* chromosomes (Fig. 3B) except for heterochromatic condensed regions (in window of Fig. 3B), while signals of SCEN repeat-sequences were observed in centromeric regions of chromosomes (Fig. 3C). Three independent studies indicated that 550-bp sequences were interspersed among *S. officinarum* chromosomes and should not be repeated at particular loci. These interspersed 550-bp sequences were named SoIS550, that is, *Saccharum officinarum* interspersed

Fig. 3. FISH of SoIS550 on *S. officinarum* cv. Badila chromosomes. (A) DAPI-stained chromosomes. (B) FISH signals of DIG-labeled SoIS550. (C) FISH signals of biotin-labeled SCEN. In windows: Enlargement of chromosomes with the heterochromatic region indicated by arrowheads. Bar indicates 5 µm.

sequences of 550-bp.

The present status of SoIS550 was investigated among six *Saccharum* species and two relative species in Table 1. PCR amplification with the primer set SCM-F and R550 yielded 550-bp products from genomic DNA of all four cultivated species, *S. officinarum*, *S. sinense*, *S. barberi*, and *S. edule*, and one wild species *S. robustum*, but did not show any products from *S. spontaneum*, *E. arundinaceus* and *Z. mays* (Fig. 2A), while rDNA sequences were amplified from all species examined (Fig. 2C). PCR amplification with the primer set SCM-F and R180 yielded 180-bp, 1.0-kbp and faint 2.0-kbp products from genomic DNA of five *Saccharum* species, *S. edule*, *S. robustum*, *S. officinarum*, *S. sinense*, and *S. barberi*, and only 180-bp products from *Z. mays*, but did not show any products from *S. spontaneum* and *E. arundinaceus* (Fig. 2B). Sizes of PCR products were almost the same among these five *Saccharum* species. Southern hybridization of DIG-labeled SoIS550 revealed three strong bands at 600-bp, 1.2-kbp and 1.7-kbp in *Alu* I-digested genomic DNA of five *Saccharum* species and a band at 800-bp in *Z. mays*, but did not give any bands in *S. spontaneum* and *E. arundinaceus* (Fig. 2D). Band patterns of Southern hybridization were almost the same among these five *Saccharum* species. These results indicated that SoIS550 was accumulated in five *Saccharum* species, but not in *S. spontaneum* and *E. arundinaceus*. The similar band patterns of PCR amplification and Southern hybridization among the five species suggested that the present status of SoIS550 should be similar among these species.

The domestication of sugar canes has been examined in various studies (Daniels and Roach 1987; Guimarães and Sobral 1998; Irvine 1999; D'Hont et al., 2002). These reports indicated three important inter-species relationships among the six *Saccharum* species, that is, (1) wild *S. robustum* would be the ancestor of the two cultivated species *S. officinarum* and *S. edule*, and *S. edule* would originate from hybridization between *S. robustum* and species in another genus, (2) the other two cultivated species *S. sinense* and *S. barberi* would be hybrids of *S. officinarum* and *S. spontaneum*, and (3) *S. robustum* would

originate from hybridization between *S. spontaneum* and species in other genus or genera (summarized in Fig. 4). The four domesticated *Saccharum* species were categorized into two groups, one consisting of *S. edule* and the other comprising *S. officinarum*, *S. sinense* and *S. barberi*. Based on these relationships, wild *S. robustum* was the common ancestral species of the four cultivated species and was key to the domestication of sugar canes, because all cultivated *Saccharum* species have inherited the genomic DNA of *S. robustum* either directly or indirectly. These relationships and the species-specific accumulation of SoIS550 in this study indicated two hypotheses for the accumulation of SoIS550 in five *Saccharum* species. The first hypothesis is that the accumulation occurred in *S. robustum* or its ancestral species in another genus, and was inherited by the four cultivated species. The second hypothesis is that the accumulation occurred in the five *Saccharum* species independently. The similarity of status suggested that the accumulation of SoIS550 occurred before the domestication of the four cultivated species, not independently. The inheritance of the *S. robustum* genome, depicted by the accumulation of SoIS550, supported inter-species relationships among the *Saccharum* species as reported (Fig. 4).

Detailed sequence analysis of SoIS550 also showed partial homology to a retrotransposon, e.g., B-box and transcribe terminator of rice *p-SINE1* (Ohtsubo et al., 2004), but other characteristic sequences required to identify a

Fig. 4. Phylogenetic relationships among the six *Saccharum* species.

SINE were not detected in SoIS550. Although the function and the origin of SoIS550 were not revealed in this study, the accumulation of many copies of SoIS550 would have affected the genomic structure of the five *Saccharum* species. Additionally, the close localization of SoIS550 to centromere-related sequences (Fig. 1A) indicated SoIS550 would have affected centromere function and genomic structure in these *Saccharum* species. The present study showed that the accumulation of SoIS550 should correlate to domestication in the genus *Saccharum*.

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